PerkinElmer

- Cytotoxicity/Cell Proliferation Assays

ATPLite 1Step

- ATPlite 1step Luminescence Assay System, 1,000 mL Assay Kit New
- ATPlite 1step Luminescence Assay System, 10 mL Assay Kit New
- ATPlite 1step Luminescence Assay System, 100 mL Assay Kit New

ATPLite™-M

- ATPlite™-M Luminescence Assay System, 10,000 Assay Kit
- ATPlite™-M Luminescence Assay System, 1000 Assay Kit
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Application Note

A cell-based DELFIA® proliferation assay for measurement of DNA synthesis in microplate format

Identification of a compund's ADME/Tox (absorption, distribution, metabolism, elimination and toxicity) profile prior to committing it to clinical trials is essential when assessing hit compunds. Cell proliferation is an important early ADME parameter when studying live cell function, particularly in cancer and drug discovery research. Proliferation assays can be used both to quantify cell proliferation in response to growth factors, cytokines, mitogens or nutrients and to analyze cytotoxic compounds such as anticancer drugs. Since cellular proliferation requires the replication of cellular DNA, methods based on DNA synthesis measurement can be used as an accurate indicator of cell growth. Traditionally, ^{3}H-thymidine has been used to label DNA. A non-isotopic alternative for ^{3}H-thymidine is 5-bromo-2'-deoxyuridine (BrdU), a pyrimidine analog, which can be incorporated into newly synthesized DNA instead of thymidine. BrdU is detected using the DELFIA® technology.

PRINCIPLES OF THE ASSAY

The DELFIA® Cell Proliferation assay is a time-resolved fluoroimmunoassay based on the incorporation of BrdU into newly synthesized DNA strands of proliferating cells cultured in microtiter plates. Incorporated BrdU is detected using a europium labelled monoclonal antibody. To allow antibody detection cells are fixed and DNA denatured using Fix Solution. Unbound antibody is washed away and DELFIA® Inducer is added to dissociate europium ions from the labelled antibody into solution, where they form highly fluorescent chelates with components of the DELFIA® Inducer (Figure 1). The fluorescence measured is proportional to the DNA synthesis in the cell population of each well. The assay can be used for the direct assessment of cell numbers, and also assay for cytotoxic effects as an endpoint measurement. The assay can be used with adherent cells as well as with cells in suspension.

Table 1. Cells used in the optimization of the assay.

Adherent Cells	Suspension Cells
CHO-K1	Human lymphocytes
HeLa	P815
HEK 293	Jurkat
	CHO-S

The assay procedure is dependent on the cell line used and exact incubation times have to be optimized for each experimental setup individually. The following assay procedure is appropriate for most applications:

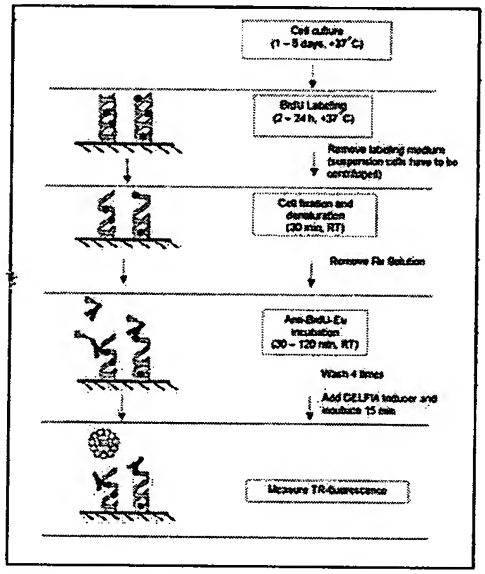

Figure 1. Assay procedure.

ASSAY PROCEDURE

1. Place appropriate amount of cells in a 96-well plate (at a final volume of 100 or 200 µL per well) and incubate them in the precence of various concentration of the test substance at 37°C in a humified 5 % CO_2 atmosphere. The incubation period depends on the cell type used. For most experimental approaches, an incubation period of 24 – 120 hours is approriate. Two kinds of controls should also be performed to insure the validity of the experimental setup. Information about the unspecific binding of BrdU and anti-BrdU-Eu is provided by blank (no cells are added to the well, only culture medium). Information about the background of the assay is provided when no BrdU is added to the wells.
2. Label cells with BrdU by adding 10 µL/well (if the cells were cultured in 100 µL) or 20 µL/well (if the cells were cultured in 200 µL) of BrdU Labeling Solution diluted in culture medium and reincubate the cells for additional 2 to 24 hours at 37°C in a humified 5 % CO_2 atmosphere.
3. Remove labeling medium. Suspension cells have to be centrifuged at 300 x g for 10 minutes before removing the labeling medium.
4. Add 100 µL/well Fix Solution and incubate for 30 minutes at room temperature.
5. Remove Fix Solution thoroughly and add 100 µL/well Anti-BrdU-Eu working solution (0.5 µg/mL) and incubate for 30 - 120 minutes at room temperature.
6. Wash 4 times using the DELFIA® Platewash.
7. Add 200 µL DELFIA Inducer directly from the reagent bottle to each well using the DELFIA® Plate Dispense or Eppendorf Multipette and shake the plate on the DELFIA® Plateshake at room temperature for 15 minutes.
8. Measure the Eu-fluorescence in a time-resolved fluorometer.

RESULTS

Titration of the number of cells

The DELFIA® Cell Proliferation assay is very sensitive, less than 100 cells per well can be detected, when incubating for just 2 h with BrdU. The dynamic range for the assay is at least 3 orders of magnitude.

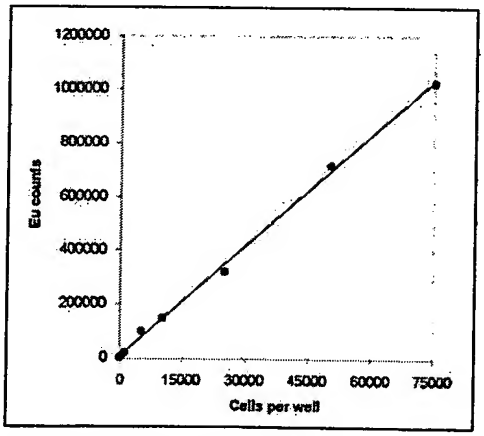

Figure 2. Suspension cells, P815 (mouse mast cells), were used for titrating the number of cells that can be detected by the assay. The cells were titrated in the microtiterplate at the concentrations indicated in the figure. After a 24 h incubation with BrdU, the incorporation was detected as described in the Assay Procedure. Note that the cells were not grown in the microtiterplate prior to the assay, so these are the actual numbers of cells per well.

The benefits of speed, convenience and sensitivity can also be applied to cytotoxicity assays where cells can be treated with for example cytotoxic agents or cytostatic drugs.

Measurement of the proliferation of mitogen-activated, human peripheral blood lymphocytes.

A typical application is the measurement of proliferation in mitogen-activated primary lymphocytes. The DELFIA® Cell Proliferation kit can be used for measurement of proliferation in primary cells. A hundred thousand cells per well were used for an assay where the cells were grown for 48 h together with the stimulant prior to labeling with BrdU (Figure 3).

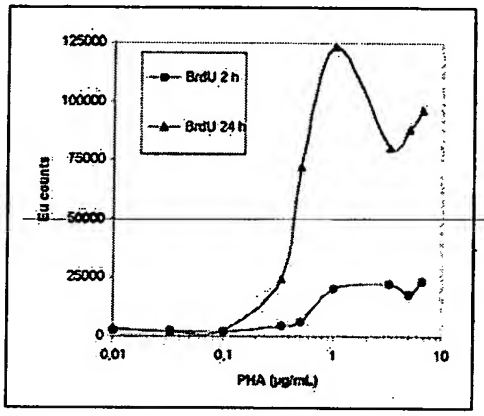

Figure 3. Human peripheral blood lymphocytes were isolated by density gradient centrifugation and cultured in microtiter plates for 48 h in the presence of various concentrations of the mitogen phytohaemagglutinin (PHA). Subsequently, BrdU was added and the cells were reincubated for an additional 2 or 24 h. BrdU incorporation was determined as described in the Assay Procedure.

Comparison of the DELFIA® Cell Proliferation assay to ^{3}H-incorporation and to a colorimetric measurement of metabolism

The DELFIA® Cell Proliferation kit has here been compared to the incorporation of tritiated thymidine and to a colorimetric proliferation assay. The tritiated thymidine method is similar to incorporation of BrdU, both based on the incorporation of a labeled nucleotide during DNA synthesis, while the colorimetric assay measures the metabolic activity of cellular enzymes.
Figure 4 compares data obtained with the DELFIA® Cell Proliferation assay, with increasing numbers of cells, to that obtained using tritiated thymidine incorporation or the colorimetric assay. The DELFIA® Cell Proliferation assay shows excellent correlation with tritiated thymidine and the colorimetric assay. R-values are typically greater than 0.98. Figure 5 shows a comparison between the BrdU and tritiated thymidine incorporation for measuring proliferation in human peripheral lymphocytes, stimulated with the mitogen PHA.

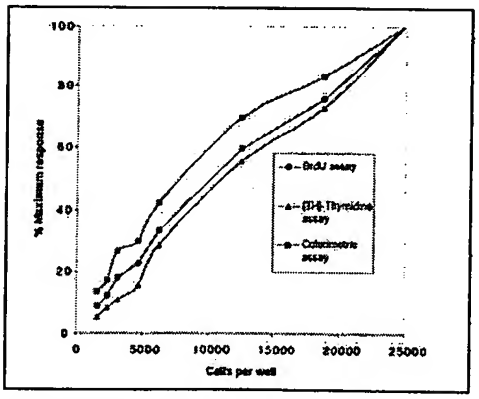

Figure 4. Comparison of DELFIA® Cell Proliferation assay, colorimetric measurement of proliferation and tritiated thymidine incorporation with increasing number of CHO-KI cells. The cells were titrated in the microtiterplate at the concentrations indicated in the figure and grown overnight. After a 2 h incubation with BrdU or tritiated thymidine, the BrdU incorporation was detected as described in the Assay Procedure. The tritiated thymidine incorporation and the colorimetric assay were performed following standard protocols.

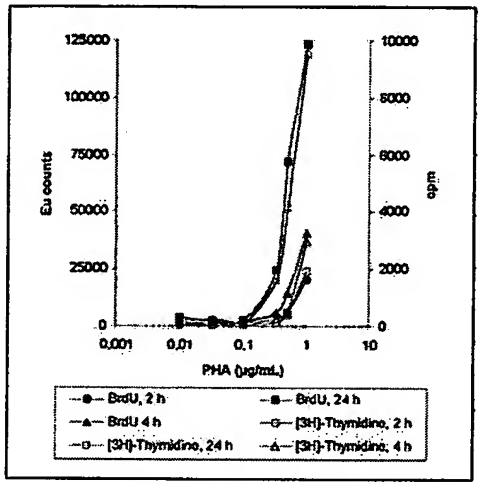

Figure 5. Comparison between DELFIA® Cell Proliferation assay and tritiated thymidine incorporation to measure the proliferative effect of PHA in human lymphocytes. Human peripheral blood lymphocytes were isolated and cultured in microtiter plates for 48 h in the presence of various concentrations of PHA. Subsequently, BrdU or tritiated thymidine were added and the cells were reincubated for an additional 2, 4 or 24 h. BrdU incorporation was determined as described in the Assay Procedure. The tritiated thymidine incorporation was performed following standard protocol.

Summary of method advantages

- **Non-radioactive:** Since the kit uses fluorescense it is completely safe. It overcomes the need for scintillation coctail and radioactive waste disposal associated with ^{3}H-Thymidine.
- **Sensitive:** Can detect as few as 100 cells per well, increased sensitivity reduces the amount of cells required per assay.
- **Works with both adherent- and suspension cells:** The method works equally well with adherent and non-adherent cells, whether they be cell lines or primary cultures.
- **Automation:** The assay is carried out at room temperature in a single microtiter plate, no transfer of cell is needed, and a large number of samples can be processed simultaneously.
- **Robust:** The signal is measurable for 8 hours at RT. A dry plate can be remeasured by adding 200 µLs of DELFIA Inducer to the well.

Products used:
* 1450-517, Wallac TC Isoplates
* AD0200, DELFIA® Cell Proliferation kit, 960 assays
* 1296-026, DELFIA® Platewash
* 1296-001/002 or 1296-003/004, DELFIA® Plateshake
* 2100, EnVision™ Multilabel Reader

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Worldwide Headquarters: PerkinElmer Life Sciences, 549 Albany Street, Boston, MA 02118-2512 USA (800) 551-2121
European Headquarters: PerkinElmer Life Sciences, Imperiastraat 8, BE-1930 Zaventem Belgium +32 2 717 7911
Technical Support: in Europe: techsupport.europe@perkinelmer.com in US and Rest of World: techsupport@perkinelmer.com
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Cell Viability/Proliferation Assays

- ATP Based Viability Assay Kits
- BrdU Cell Proliferation Assay Kits
- Cell Viability / Proliferation Assays
- Cell Viability/Proliferation Assay Reagents
  - Alamar Blue
  - Brd U
  - Cell Viability/Proliferation Assay Reagents
  - MTT
  - Trypan Blue
  - XTT
- Comparative Genomic Hybridization (CGH)
- LDH Cytotoxicity Assays
- Live Cell Labeling Kits
- Micronucleii Assay Kits
- MTT Cell Proliferation Assay Kits
- Phagocytosis Assay Kits
- Telomere Length & TRAP Assays
- Thymidine Uptake Assay Kits

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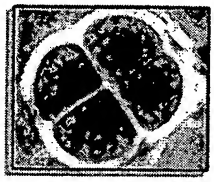

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Cell Viability/Proliferation Assays New Technologies:

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BacTiter-Glo™ Microbial Cell Viability Assay

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Technical Bulletin No. 337

INSTRUCTIONS FOR USE OF PRODUCTS G8230, G8231, G8232, AND G8233.
All technical literature is available on the Internet at www.promega.com
Please visit the web site to verify that you are using the most current version of this Technical Bulletin.

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I. Description

The BacTiter-Glo™ Microbial Cell Viability Assay[a,b] is a homogeneous method for determining the number of viable bacterial cells in culture based on quantitation of the ATP present. ATP is an indicator of metabolically active cells. The BacTiter-Glo™ Assay is designed for either single-use or multiwell-plate formats for high-throughput screening (HTS). The homogeneous assay procedure involves adding a single reagent (BacTiter-Glo™ Reagent) directly to bacterial cells in medium and measuring luminescence (Figure 1). Washing cells, removing culture medium and performing multiple pipetting steps are not required.

The formulation of the reagent supports bacterial cell lysis and generation of a luminescent signal in a homogeneous "add, mix, measure" format. The luminescent signal is proportional to the amount of ATP present, which is directly proportional to the number of cells present in culture (Figure 2). The BacTiter-Glo™ Reagent relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) and a proprietary formulation for extracting ATP from bacteria. The BacTiter-Glo™ Assay generates a "glow-type" luminescent signal, produced by the luciferase reaction shown in Figure 3, which has a signal half-life generally over 30 minutes depending on the bacterium and medium. The assay has been shown to detect a variety of bacteria, yeast and fungi (Table 1). The homogeneous format reduces pipetting errors that may be introduced during the multiple steps required by other methods of ATP measurement.

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Advantages

- Simplify your Assay: The add, mix, measure format reduces the number of handling steps to fewer than that required for similar ATP assays, no injectors required.
- Get Results Quickly: Data can be recorded 5 minutes after adding and mixing Reagent, and sensitivity allows you to detect growth sooner.
- Increase your Sensitivity: Measures ATP from as few as 10 bacterial cells.
- Choose your Format: Can be used with various multiwell or single-use formats. Data can be recorded by luminometer or CCD camera.
- Achieve Robust Signal: Luminescent signal is stable, with a 30-minute half-life.

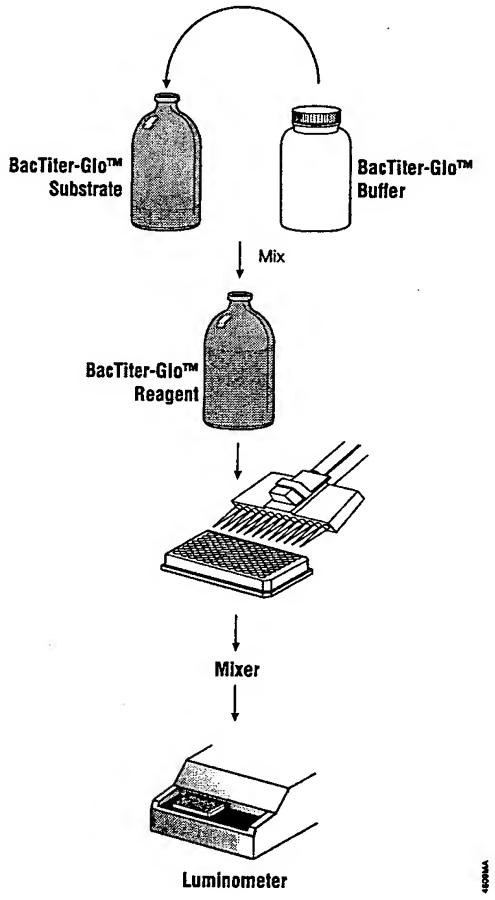

Figure 1. Diagram of the BacTiter-Glo™ Microbial Cell Viability Assay protocol. The assay is suitable for single-tube or multiwell-plate formats shown here.

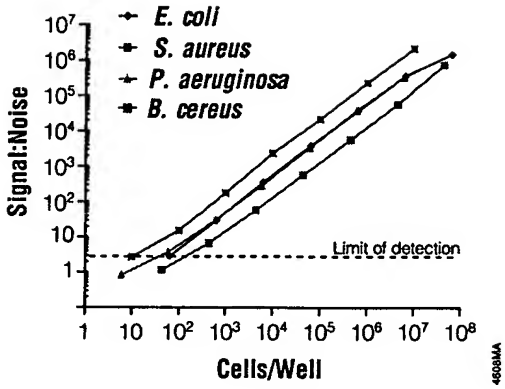

Figure 2. Bacterial cell numbers correlate with luminescent signal. Four bacterial strains *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923), *Pseudomonas aeruginosa* (ATCC27853) and *Bacillus cereus* (ATCC10987) were grown in Mueller Hinton II (MH II) Broth (BD Cat.# 297963; see Section IV for growth medium recommendations) at 37°C overnight. The overnight culture was diluted 50-fold in fresh MH II Broth and then incubated for several hours to reach log phase. Samples of the culture were serially diluted using MH II Broth in a 96-well plate. The assay was performed according to the protocol described in Section III. The reconsituted BacTiter-Glo™ Reagent was equilibrated for 1.5 hours at room temperature to achieve better sensitivity (see Reagent Background in Section IV). Luminescence was recorded on a Veritas™ Microplate Luminometer from Turner Biosystems (Cat.# E6501). Signals represent the mean of three replicates for each measurement. Bacterial cell numbers were determined by plate counting of colony forming units on Luria-Bertani agar plates. The signal-to-noise ratio was calculated: S:N = [mean of signal–mean of background]/standard deviation of background]. There is a linear correlation between luminescent signal and the number of cells over five orders of magnitude. The limits of detection drawn from this experiment for *E. coli, S. aureus, P. aeruginosa* and *B. cereus* are approximately 40, 150, 70 and 10 cells, respectively.

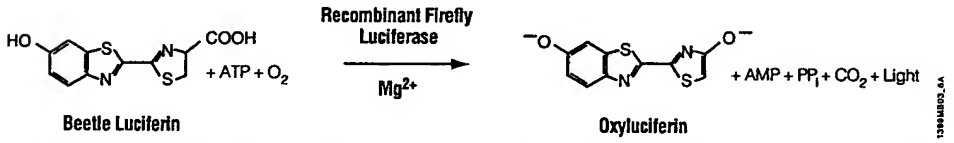

Figure 3. The luciferase reaction. Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg^{2+}, ATP and molecular oxygen.

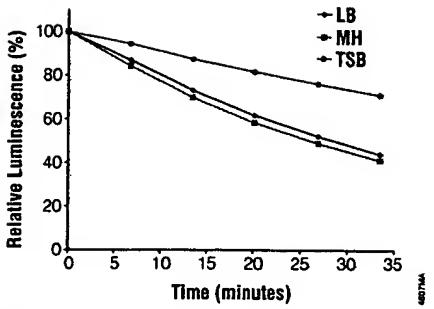

Figure 4. BacTiter-Glo™ Reagent generates a glow-type luminescent signal. *E. coli* cells were grown and assayed as described in Figure 2. Three media were tested: Luria-Bertani Broth, Mueller Hinton II (MH II) Broth (BD Cat.# 297963), and Trypticase Soy Broth (TSB, BD Cat.# 299113). Approximately 10^6 *E. coli* cells were used for the assay. The stability of the luminescence signal was monitored over time. Luminescence was recorded on a Veritas™ Microplate Luminometer from Turner Biosystems (Cat.# E6501). The half-lives of the lumine scence signals in MH II, LB and TSB were 26, 28 and 68 minutes, respectively.

II. Product Components

Product	Size	Cat.#
BacTiter-Glo™ Microbial Cell Viability Assay	10ml	G8230

For Laboratory Use. Substrate is sufficient for 100 assays at 100µl/assay in 96-well plates or 400 assays at 25µl/assay in 384-well plates. Includes:

- 10ml BacTiter-Glo™ Buffer
- 1 vial BacTiter-Glo™ Substrate (lyophilized)
- 1 Protocol

Product	Size	Cat.#
BacTiter-Glo™ Microbial Cell Viability Assay	10 x 10ml	G8231

For Laboratory Use. Each vial of Substrate is sufficient for 100 assays at 100µl/assay in 96-well plates or 400 assays at 25µl/assay in 384-well plates. Includes:

- 10 x 10ml BacTiter-Glo™ Buffer
- 10 vials BacTiter-Glo™ Substrate (lyophilized)
- 1 Protocol

Product	Size	Cat.#
BacTiter-Glo™ Microbial Cell Viability Assay	100ml	G8232

For Laboratory Use. Substrate is sufficient for 1,000 assays at 100µl/assay in 96-well plates or 4,000 assays at 25µl/assay in 384-well plates. Includes:

- 100ml BacTiter-Glo™ Buffer
- 1 vial BacTiter-Glo™ Substrate (lyophilized)
- 1 Protocol

Product	Size	Cat.#
BacTiter-Glo™ Microbial Cell Viability Assay	10 x 100ml	G8233

For Laboratory Use. Each vial of substrate is sufficient for 1,000 assays at 100µl/assay in 96-well plates or 4,000 assays at 25µl/assay in 384-well plates (10,000 to 40,000 total assays). Includes:

- 10 x 100ml BacTiter-Glo™ Buffer
- 10 vials BacTiter-Glo™ Substrate (lyophilized)
- 1 Protocol

Storage Conditions: For long-term storage, the lyophilized BacTiter-Glo™ Substrate and BacTiter-Glo™ Buffer should be stored at –20°C. The unopened BacTiter-Glo™ Substrate and Buffer are stable for six months when stored at –20°C. For frequent use, the BacTiter-Glo™ Buffer can be stored at 4°C or at room temperature for 48 hours without loss of activity. For optimal performance, reconstituted BacTiter-Glo™ Reagent (buffer plus substrate) should be used within eight hours when the reagent is kept at room temperature. The reconstituted BacTiter-Glo™ Reagent can be stored at 4°C for four days, at –20°C for one week or at –70°C for one month with less than 20% loss of activity.

III. Protocol for Performing the BacTiter-Glo™ Assay

Materials to Be Supplied by the User

- opaque-walled multiwell plates
- multichannel pipette or automated pipetting station for delivering reagent
- plate shaker or other device for mixing contents of multiwell plates
- luminometer (e.g., Veritas™ Microplate Luminometer [Cat.# E6501] or Turner TD-20/20 luminometer [Cat.# E2041, E2051]) or CCD camera capable of reading multiwell plates
- optional: ATP for generating a standard curve

Caution.
...Skin contains ATP. Because this assay is so sensitive, we recommend wearing gloves to avoid contamination.

A. Reagent Preparation

1. Thaw the BacTiter-Glo™ Buffer and equilibrate to room temperature before use. For convenience the BacTiter-Glo™ Buffer may be thawed and stored at room temperature for up to 48 hours before use.
2. Equilibrate the lyophilized BacTiter-Glo™ Substrate to room temperature before use.
3. Transfer the appropriate volume (10ml for Cat.# G8230, G8231 or 100ml for Cat.# G8232, G8233) of BacTiter-Glo™ Buffer into the amber bottle containing BacTiter-Glo™ Substrate to reconstitute the lyophilized enzyme/substrate mixture. This forms the BacTiter-Glo™ Reagent.
4. Mix by gently vortexing, swirling or by inverting the bottle to obtain a homogeneous solution. The BacTiter-Glo™ Substrate should go into solution easily, in less than one minute.
5. Equilibrate Reagent at room temperature for at least 15 minutes.

Equilibrate
...the reagent at room temperature for at least 15 minutes. To achieve maximum sensitivity, additional equilibration time may be required. See "Reagent Background" in Section IV.B for more information.

B. Protocol for Measuring ATP From Bacteria

Note: All steps are performed at room temperature (22–25°C).

1. Prepare an opaque-walled multiwell plate with microbial cells in culture medium (e.g., 100µl for each well of a 96-well plate or 25µl for each well of a 384-well plate).
2. Prepare control wells containing medium without cells to obtain a value for background luminescence.
3. Equilibrate the plate and its contents to room temperature.
4. Add a volume of BacTiter-Glo™ Reagent equal to the volume of cell culture medium present in each well (e.g., add 100µl of reagent to 100µl of medium containing cells for the 96-well plate format or 25µl of reagent for the 384-well plate format).

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5. Mix contents briefly on an orbital shaker and incubate for five minutes.
6. Record luminescence.

Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.

C. Protocol for Generating an ATP Standard Curve (optional)

Note: All steps are performed at room temperature (22–25°C).

1. Prepare 1µM ATP in culture medium (100µl of 1µM ATP solution contains 10–10 moles ATP).
2. Prepare 10-fold serial dilutions of ATP in culture medium (1µM to 10pM; 100µl volumes would contain 10^{-10} to 10^{-15} moles of ATP).
3. Prepare a multiwell plate with varying concentrations of standard ATP solution in 100µl medium.
4. Add a volume of BacTiter-Glo™ Reagent equal to the volume of ATP standard present in each well (1:1 ratio).
5. Mix contents briefly on an orbital shaker and incubate for one minute. Since there is no lysis required to release ATP, longer incubations are not required.
6. Record luminescence.

IV. Appendix

A. Overview of the BacTiter-Glo™ Assay

The assay system utilizes a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) to enable extraction of ATP from bacterial cells and to support a stable "glow-type" luminescent signal. Historically, firefly luciferase purified from *Photinus pyralis* has been used in reagents for ATP assays (1–3). However, this enzyme has only moderate stability in vitro and is sensitive to factors such as pH and detergents, limiting its usefulness in a robust homogeneous ATP assay. Promega has successfully developed a stable form of luciferase (Ultra-Glo™ Recombinant Luciferase) based on the gene from another firefly, *Photuris pennsylvanica*, using an approach to select for characteristics that improve performance in ATP assays (4). In addition, we developed a proprietary formulation to achieve rapid and more efficient extraction of ATP from a variety of microbial cells (Table 1). The combination of these two essential elements in the BacTiter-Glo™ Reagent enabled design of a homogeneous single-reagent system for performing ATP assays on cultured cells. The reagent is physically robust and provides a sensitive and stable luminescent output.

Table 1. BacTiter-Glo™ Reagent Works with a Variety of Microbial Organisms.

Gram⁻ bacterium	Gram⁺ bacterium	Others
Escherichia coli	*Staphylococcus aureus*	*Saccharomyces cerevisiae*
Pseudomonas aeruginosa	*Enterococcus faecalis*	*Candida albicans*
Enterobacter cloacae	*Streptococcus pneumoniae*	
Flavobacterium okeanokoites	*Bacillus subtilis*	
Haemophilus influenzae	*Bacillus cereus*	
Proteus vulgaris	*Arthrobacter luteus*	
Salmonella typhimurium		
Yersinia enterocolitica		
Francisella philomiragia		

B. Additional Considerations

Temperature: The intensity and rate of decay of the luminescent signal from the BacTiter-Glo™ Assay depend on the rate of the luciferase reaction. Environmental factors that affect the rate of the luciferase reaction will result in a change in the intensity of light output and the stability of the luminescent signal. Temperature is one factor that affects the rate of this enzymatic assay and thus the light output. For consistent results, equilibrate assay plates to room temperature before performing the assay. Insufficient equilibration may result in a temperature gradient effect between the wells in the center and on the edge of the plates.

Growth Medium: Growth medium is another factor that could contribute to the background luminescence and affect the luciferase reaction in terms of signal level and signal stability (Figure 4). We have used MH II Broth (cation adjusted Mueller Hinton Broth; Becton, Dickinson and Company Cat.# 297963) for all our experiments unless otherwise mentioned. It supports growth for most commonly encountered aerobic and facultative anaerobic bacteria and is selected for use in food testing and antimicrobial susceptibility testing by Food and Drug Administration and National Committee for Clinical Laboratory Standards (NCCLS) (5,6). MH Medium has low luminescence background and good batch-to-batch reproducibility.

Chemicals: The chemical environment of the luciferase reaction will affect the enzymatic rate and thus luminescence intensity. Solvents used for the various chemical compounds tested for their antimicrobial activities may interfere with the luciferase reaction and thus the light output from the assay. Interference with the luciferase reaction can be determined by assaying a parallel set of control wells containing medium without cells. Dimethylsulfoxide (DMSO), commonly used as a vehicle to solubilize organic chemicals, has been tested at final concentrations up to 2% in the assay and has little effect on light output (<5% loss of activity).

Plate and Tube Recommendations: The BacTiter-Glo™ Assay is suitable for multi-well-plate or single-tube formats. Standard opaque-walled multiwell plates suitable for luminescence measurements are recommended for use. Opaque-walled plates with clear bottoms allowing microscopic visualization of cells also may be used; however, these plates will have diminished signal intensity and greater cross-talk between wells. Opaque white tape may be used to reduce luminescence loss and cross-talk. For single-tube assays, the standard tube accompanying the luminometer used (e.g., Turner BioSystems TD-20/20 luminometer) should be suitable.

Cellular ATP Content: Different bacteria have different amounts of ATP per cell, and values reported for the ATP level in cells vary considerably (7,8). Factors that affect the ATP content of cells such as growth phase, medium, and presence of metabolic inhibitors, may affect the relationship between cell number and luminescence (7).

Mixing: Optimum assay performance is achieved when the BacTiter-Glo™ Reagent is completely mixed with the sample of cultured cells. For all of the bacteria we tested, maximum luminescent signals were observed after efficiently mixing and incubating for 1–5 minutes. However, complete extraction of ATP from certain bacteria, yeast or fungi may take longer. Automated pipetting devices using a greater or lesser force of fluid delivery may affect the degree of subsequent mixing required. Ensure complete reagent mixing in 96-well plates by using orbital plate shaking devices built into many luminometers. We recommend considering these factors when performing the assay and determining whether a mixing step and/or longer incubation is necessary.

Reagent Background: Despite the rigorous ATP-free manufacturing process, a trace amount of ATP is still present in the BacTiter-Glo™ Substrate and Buffer. In addition, ATP could be introduced by the user during the reconstitution step. When the BacTiter-Glo™ Substrate and Buffer are mixed together to reconstitute BacTiter-Glo™ Reagent, a background luminescence signal is generated that decreases over time as the ATP is being consumed. This process is referred to as "burn-off." Complete burn-off to the lowest achievable background could take up to two hours. However, this is only necessary when the maximum sensitivity is required (e.g., detection of very low number of microorganisms).

C. Examples of BacTiter-Glo™ Assay Applications

The BacTiter-Glo™ Assay provides a simple and robust way to quantify bacteria with superb sensitivity and dynamic range. Some examples of its applications are shown below.

Screening for Antimicrobial Compounds

We used the BacTiter-Glo™ Assay to screen one rack of Library of Pharmacologically Active Compounds from Sigma (LOPAC, #8, enzyme inhibitors, total of 80 compounds) for antimicrobial activity against *Staphylococcus aureus*. The results are shown in Figure 5. All positive controls of standard antibiotics (boxed points) and three LOPAC compounds (circled points) exhibited signficant anti-*S. aureus* activity. The three LOPAC hits were D6, emodin; D11, sanguinarine chloride; and H7, minocycline. Their anti-*S. aureus* activities were reported in the literature (9–11).

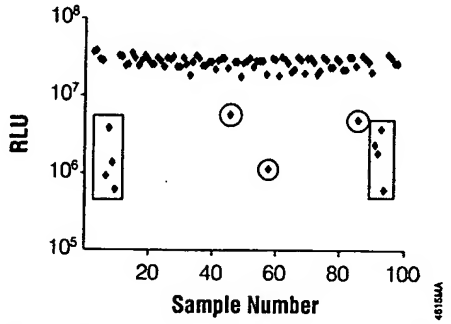

Figure 5. Screening for antimicrobial compounds using the BacTiter-Glo™ Assay. *S. aureus* ATCC 25923 strain was grown in Mueller Hinton II (MH II) Broth (BD Cat.# 297693; see Section IV for growth medium recommendations) at 37°C overnight. The overnight culture was diluted 100-fold in fresh MH II Broth and used as inoculum for the antimicrobial screen. Working stocks (50X) of LOPAC compounds and standard antibiotics were prepared in DMSO. Each well of the 96-well multiwell plate contained 245µl of the inoculum and 5µl of the 50X working stock. The multiwell plate was incubated at 37°C for 5 hours. One hundred microliters of the culture was taken from each well, and the BacTiter-Glo™ Assay was performed according to the protocol described in Section III. Luminescence was measured using a Veritas™ Microplate Luminometer from Turner Biosystems (Cat.# E6501). The samples and concentrations are: Wells 1–4 and 93–96, negative control of 2% DMSO, wells 5–8 and 89–92, postive controls of 32µg/ml standard antibiotics tetracycline, ampicillin, gentamicin, chloramphenicol, oxacillin, kanamycin, piperacillin, and erythromycin; wells 9–88, LOPAC compounds at 10µM.

Evaluating antimicrobial compound activity

We examined the dosage effects of oxacillin on *S. aureus* using the BacTiter-Glo™ Assay. The results are shown in Figure 6. Oxacillin showed anti-*S.aureus* activity in a dosage-dependent fashion. The reported and observed minimal inhibitory concentration (MIC) values for oxacillin on *S. aureus* ATCC 25923 in cation-adjusted MH II Broth are 0.125–0.5µg/ml (6), corresponding to approximately IC_{75}–IC_{90} values on the dosage curve determined using the BacTiter-Glo™ Assay.

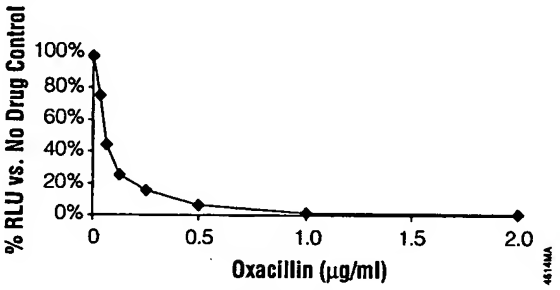

Figure 6. Evaluating antimicrobial compounds using the BacTiter-Glo™ Assay. *S. aureus* ATCC 25923 strain and oxacillin were prepared as described in Figure 5 and incubated at 37°C; the assay was performed after 19 hours of incubation as recommended for MIC determination by NCCLS (6). The relative percentage of RLU compared to the no-oxacillin control is shown. Luminescence was recorded on a Veritas™ Microplate Luminometer from Turner Biosystems (Cat.# E6501).

Examining Bacterial Growth with Extended Sensitivity and Range

We examined the growth of *E. coli* using either the BacTiter-Glo™ Assay or optical density (O.D.) measurement. The results are shown in Figure 7. The extended sensitivity and range of the BacTiter-Glo™ Assay allows users to monitor *E. coli* growth immediately after inoculation. When measuring growth by O.D., the first significant measurement (0.025) did not occur until 5 hours after inoculation. The growth curve determined by the BacTiter-Glo™ Assay has a dynamic range over six orders of magnitude compared to the growth curve determined by O.D. measurement, which only has a range of about two orders of magnitude. The increased dynamic range allows researchers to more easily monitor slow growing bacteria.

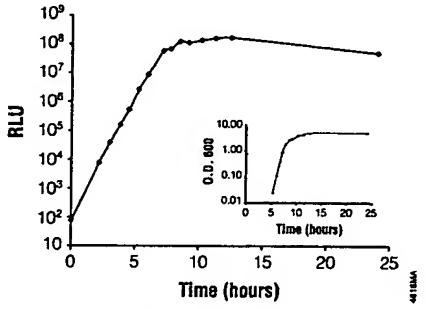

Figure 7. Evaluating bacterial growth using the BacTiter-Glo™ Assay. *E. coli* ATCC 25922 strain was grown in Mueller Hinton II (MH II) Broth (B.D. Cat.# 297963); see Section IV for growth medium recommendations) at 37°C overnight. The overnight culture was diluted 1:10^6 in 50ml of fresh MH II Broth and incubated at 37°C with shaking at 250rpm. Samples were taken at various time points, and the BacTiter-Glo™ Assay was performed according to the protocol described in Section III. Luminescence was recorded on a Veritas™ Microplate Luminometer. Optical density was measured at 600nm (O.D. 600) using a Beckman DU650 spectrophotometer. Diluted samples were used when readings of RLU and O.D. exceeded 10^8 and 1, respectively.

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E. Related Products

Luminometers

Product	Size	Cat.#
Veritas™ Microplate Luminometer	1 each	E6501
Turner BioSystems Luminometer Model TD-20/20 Genetic Reporter Instrumentation Package for Stabilized Assays	1 each	E2041
Turner BioSystems Luminometer Model TD-20/20 Genetic Reporter Instrumentation Package for Stabilized Assays with Printer	1 each	E2051
Disposable Polyproylene Test Tubes	1,000 tubes	E4221

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Cell Viability Assays

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay[a,b] (luminescent, ATP)	10ml	G7570
	10 x 10ml	G7571
	100ml	G7572
	10 x 100ml	G7573
CellTiter-Blue™ Cell Viability Assay (colorimetric, resazurin)	20ml	G8080
	100ml	G8081
	10 x 100ml	G8082
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay*[c] (colorimetric, MTS)	200 assays	G3582
	1,000 assays	G3580
	5,000 assays	G3581
CytoTox-ONE™ Homogeneous Membrane Integrity Assay[d] (LDH)	200–800 assays	G7890
	1,000–4,000 assays	G7891
CytoTox-ONE™ Homogeneous Membrane Integrity Assay, HTP[d]	1,000–4,000 assays	G7892

*For Laboratory Use.

Apoptosis Assays

Product	Size	Cat.#
Caspase-Glo™ 3/7 Assay System*[a,b]	2.5ml	G8090
	10ml	G8091
	100ml	G8092
Caspase-Glo™ 8 Assay*[a,b]	2.5ml	G8200
	10ml	G8201
	100ml	G8202
Caspase-Glo™ 9 Assay*[a,b]	2.5ml	G8210
	10ml	G8211
	100ml	G8212
Apo-ONE® Homogeneous Caspase-3/7 Assay	1ml	G7792
	10ml	G7790
	100ml	G7791

*For Laboratory Use.

[a]U.S. Pat. No. 6,602,677 and Australian Pat. No. 754312 have been issued to Promega Corporation for thermostable luciferases and methods of production. Other patents are pending.

[b]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

[c]The MTS tetrazolium compound is the subject of U.S. Pat. No. 5,185,450 assigned to the University of South Florida and is licensed exclusively to Promega Corporation.

[d]Patent Pending.

Promega Corporation
2800 Woods Hollow Road
Madison, WI 53711-5399 USA
Telephone 608-274-4330
Fax 608-277-2516
Internet www.promega.com
ISO 9001 Certified

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BacTiter-Glo™ Microbial Cell Viability Assay: *Experienced User's Protocol*

This quick protocol is intended as an easy-to-follow reminder for experienced users. Please follow the complete protocol (Sections III.A through III.B) the first time you use the BacTiter-Glo™ Microbial Cell Viability Assay.

Reagent Preparation (Section III.A)	1. Thaw the BacTiter-Glo™ Buffer and equilibrate to room temperature before use. For convenience the BacTiter-Glo™ Buffer may be thawed and stored at room temperature for up to 48 hours before use. 2. Equilibrate the lyophilized BacTiter-Glo™ Substrate to room temperature before use. 3. Transfer the appropriate volume (10ml for Cat.# G8230 and G8231 or 100ml for Cat.# G8232 and G8233) of BacTiter-Glo™ Buffer into the amber bottle containing BacTiter-Glo™ Substrate to reconstitute the lyophilized enzyme/substrate mixture. This forms the BacTiter-Glo™ Reagent. 4. Mix by gently vortexing, swirling or by inverting the bottle to obtain a homogeneous solution. The BacTiter-Glo™ Substrate should go into solution easily, in less than one minute. 5. Equilibrate Reagent at room temperature for at least 15 minutes.
Measuring ATP from Bacteria (Section III.B)	Note: All steps are performed at room temperature. 1. Prepare an opaque-walled multiwell plate with microbial cells in culture medium (e.g., 100µl for each well of a 96-well plate or 25µl for each well of a 384-well plate). 2. Prepare control wells containing medium without cells to obtain a value for background luminescence. 3. Equilibrate the plate and its contents to room temperature. 4. Add a volume of BacTiter-Glo™ Reagent equal to the volume of cell culture medium present in each well (e.g., add 100µl of Reagent to 100µl of medium containing cells for the 96-well plate format). 5. Mix contents briefly on an orbital shaker and incubate for five minutes. 6. Record luminescence. **Note:** Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.

Definitions of **inorganic compound** on the Web:

- Combination of two or more elements other than those used to form organic compounds. See organic compound.
  ilrdss.sws.uiuc.edu/glossary/glossary_allresults.asp

- Compounds that contain no carbon or contain only carbon bound to elements other than hydrogen.
  www.sludgehappens.com/dewatering_glossary.htm

- any compound that does not contain carbon
  wordnet.princeton.edu/perl/webwn

- An inorganic compound is a chemical compound not containing carbon. However, elemental carbon (diamond or graphite) as well as carbon monoxide, carbon dioxide and carbonates are typically considered inorganic, while methane, ethanol and similar simple hydrocarbons are referred to as organic compounds.
  en.wikipedia.org/wiki/Inorganic_compound

Definitions of **cell proliferation** on the Web:

- An increase in the number of cells as a result of cell growth and cell division.
  www.cancerhub.info/reference/glossary.aspx

- Increase in cell number by division.
  www.soyfacts.com.au/glossary.html

- The term cell growth is used in two different ways in biology. When used in the context of reproduction of living cells the phrase "cell growth" is shorthand for the idea of "growth in cell numbers by means of cell reproduction." During cell reproduction one cell (the "parental" cell) divides to produce daughter cells. In other contexts, "cell growth" refers to increases in cell size.
  en.wikipedia.org/wiki/Cell_proliferation

nature

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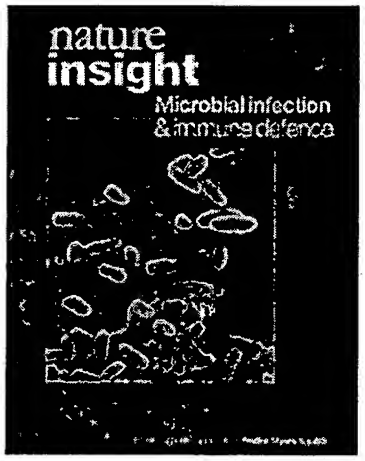
Microbial infection and immune defence

Vol. 406, No. 6797 (17 August 2000).
| PDF (190 K) |



Cover illustration
Scanning electron micrograph of *Salmonella enteritidis*, rod-shaped bacteria that cause salmonellosis.
(Image courtesy of Dennis Kunkel.)

Despite the extensive use of antibiotics and vaccination programmes, infectious diseases continue to be a leading cause of morbidity and mortality worldwide. Widespread antibiotic resistance, the emergence of new pathogens in addition to the resurgence of old ones, and the lack of effective new therapeutics exacerbate the problems.

The new approaches that are needed to deal with this increasing threat will come from the integration of two of the most active areas of biomedical research: the molecular and cellular basis of microbial pathogenesis, and the nature and manipulation of immune defence. In this month's Insight, we examine how bacteria attack and survive in the host, the mechanisms that the host uses to defend itself, and the therapeutic strategies that can be used to buttress these defences. As this is such a large and complex topic, we focus on bacterial disease; important health hazards such as HIV infection and malaria will be addressed in future Insights.

Individual articles discuss the changing patterns of infectious disease, strategies by which enteric pathogens establish infection, and the sophisticated methods that bacteria use to combat antibiotics. The innate immune system constitutes the first line of defence against infectious disease, and two articles describe the triggering of Toll-like receptors on the surface of extracellular bacteria and the ability of mycobacterial lipids to trigger T-cell responses through presentation on non-classical class I molecules. The final two papers explore the generation of vaccines against intracellular pathogens and how microbial genome sequencing will offer new approaches to the treatment and diagnosis of infectious disease.

We are pleased to acknowledge the financial support of Bristol-Myers Squibb in producing this Insight. As always, though, *Nature* carries the sole responsibility for all editorial content and peer-review. We hope that both general readers as well as experts in the field will find these articles useful and informative.

Ursula Weiss Senior Editor

nature insight

overview

On the particularity of pathogens 760
BARRY R. BLOOM
In the elemental struggle between pathogenic microbes and the immune system of the host, each strives for a unique advantage and thus each exploits its own unique particularities in pathogenesis and protection. And each presumably selects for the diversity that generally characterizes the wide range of successful host–pathogen interactions.
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Pathogenic strategies of enteric bacteria 768
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